



Peracetic acid is effective for controlling fungus on channel catfish eggs

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Abstract

Peracetic acid (PAA) is a relatively new compound suggested for use to treat pathogens in aquaculture. It is approved for use in Europe, but not in the United States. This study determined the effectiveness of PAA for fungus control on channel catfish, *Ictalurus punctatus* (Rafinesque), eggs. The study consisted of five PAA concentrations (2.5, 5, 10, 15 and 20 mg L⁻¹) and an untreated control in a flow-through system. A single spawn was used for each replication ($N = 4$). Eggs were treated twice daily until the embryos developed eyes. When hatching was complete for all viable eggs, fry were counted to determine the percent survival in each treatment. Fungal growth was severe in the untreated controls resulting in 11% survival. Treatments of 2.5, 5 and 10 mg L⁻¹ PAA were significantly different from the controls ($P < 0.05$). The highest percent survival of hatched fry was with 5 mg L⁻¹ PAA administered twice daily; the 2.5 mg L⁻¹ PAA treatment had slightly less survival, but gives a higher margin of safety in case of treatment error. Very little fungus was present in treatments receiving 2.5 mg L⁻¹ PAA or higher, and concentrations of 15 and 20 mg L⁻¹ PAA were toxic to the eggs. The mean survivals in the 0, 2.5, 5, 10, 15 and 20 mg L⁻¹ PAA treatments were 11%, 60%, 63%, 62%, 32% and 0%, respectively. Therefore, PAA may be a compound that merits further investigations regarding its use in U.S. aquaculture.

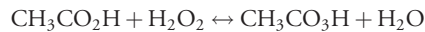
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Introduction

Fungal infections, such as saprolegniasis (*Saprolegnia* spp. Kütz), of hatchery-reared catfish eggs can cause serious losses to spawns held in hatcheries (Avery & Steeby 2004; Steeby & Avery 2005). Unfertilized eggs are easily colonized by fungus and provide nutrition for fungal growth, which can spread to healthy eggs. The Harry K. Dupree, Stuttgart National Aquaculture Research Center (SNARC), has developed a unique test system and has the proper water quality and conditions to research the effectiveness of various therapeutants to control fungus on catfish eggs in a setting that closely replicates a commercial hatchery. Compounds previously evaluated in this system include copper sulphate (CuSO₄), diquat (C₁₂H₁₂N₂Br₂), formalin (CH₃OH) and hydrogen peroxide (H₂O₂) (Mitchell *et al.* 2009; Straus *et al.* 2009a,b; Mitchell *et al.* 2010; Straus *et al.* 2011).

Peracetic acid (PAA; CH₃CO₃H) is an antimicrobial disinfectant registered by the US Environmental Protection Agency for use in agriculture, food processing and medical facilities (USEPA 2007). In Europe, PAA is also approved for use in veterinary medicine (Lehmann 1974; Schäperclaus 1991) and is one of the very few compounds approved for use in aquaculture as a disinfectant. It is the peroxide of acetic acid (CH₃CO₂H) and is commercially available in an equilibrium mixture with acetic acid, hydrogen peroxide and water. The mixture is represented by the following equilibrium:



Acetic acid + hydrogen peroxide \leftrightarrow peracetic acid + water

Although hydrogen peroxide is a disinfectant and contributes to the disinfection power of the PAA mixture, PAA is a far more potent antimicrobial agent than hydrogen peroxide (Block 1991) because of its fat solubility. It is rapidly active at low concentrations against a wide spectrum of microorganisms (Kitis 2004). The combination of PAA and hydrogen peroxide has been found to be synergistic (Alasri *et al.* 1992); it is bactericidal at 0.001%, fungicidal at 0.003%, sporicidal at 0.3% (Greenspan & MacKellar 1951) and virucidal at 0.75% (Baldry & French 1989). Peracetic acid is primarily degraded by chemical oxidation in contrast to the microbial breakdown of hydrogen peroxide by catalase activity (Block 1991). It has also been shown that PAA produces little to no toxic/mutagenic by-products after reaction with organic material (Baldry & Fraser 1988; Monarca *et al.* 2002), and it has very low environmental impact considerations.

The successful use of Wofasteril (a PAA product produced by KESLA PHARMA WOLFEN GmbH) as a treatment against fish pathogens was suggested by Schäperclaus (1991) and has recently been investigated on several pathogens (Rintamäki-Kinnunen *et al.* 2005; Meinelt *et al.* 2007a,b, 2009; Straus & Meinelt 2009; Sudová *et al.* 2010; Marchand *et al.* in press). Most of this research was carried out on *Ichthyophthirius multifiliis* (Fouquet) and recently on *Flavobacterium columnare* (Davis) and *Saprolegnia parasitica* (Humphrey). These results suggest PAA could be used for treatments in freshwater aquaculture, but more research is needed to evaluate its usefulness on various diseases under practical farming conditions as opposed to *in vitro* studies.

To facilitate a safe and fully sustainable implementation of any therapeutant, an array of new information about the compound is required (Pedersen *et al.* 2009). Therefore, this study was performed to determine whether PAA is a safe and effective treatment to control saprolegniasis on catfish eggs.

Materials and methods

Four experimental hatching troughs constructed of aluminium and divided into nine compartments (50 × 35 × 20 cm) held 35 L of water as previously described (Mitchell *et al.* 2009, 2010; Straus *et al.* 2009a,b, 2011). Filtered well water (75-µM

canister filter) flowed through the compartments continuously. Flow rate allowed for a 28-min water exchange, as in a typical hatchery, under 12 h light/12 h dark conditions with fluorescent lighting. Each compartment contained a water inlet, a stand-pipe outlet covered with a deep-water release and a plastic mesh basket to hold eggs until hatching. Aeration was achieved via rotating plastic paddles in each compartment attached to a shaft the length of the trough and turned by an electric motor.

Total alkalinity and total hardness (as CaCO_3) were measured by titration methods (Eaton & Franson 2005), and the pH was measured with an Orion Research 720A Meter (Thermo Electron Corporation). Dissolved oxygen (DO) and water temperature were monitored daily with a YSI Pro20 DO meter equipped with a polarographic sensor (YSI Environmental).

Four egg masses (<24 h old) were collected from channel catfish, *Ictalurus punctatus* (Rafinesque), broodstock spawned at SNARC. Each egg mass was gently divided into six similar weight portions (~91 g), placed into mesh baskets held in individual compartments of a single trough and acclimatized for 1 h. Three smaller samples (~12 g) from each spawn were weighed, and the adhesive matrix was dissolved with 1.5% sodium sulphite (Sigma Chemical Co.) to count the eggs; this count was used to estimate the number of eggs in each larger portion from the same spawn.

A 35 g L⁻¹ PAA stock solution was prepared with deionized water prior to treatment with VigorOx[®] SP-15 Antimicrobial Agent (FMC Corporation). VigorOx[®] SP-15 is composed of 15% PAA, 10% hydrogen peroxide and 75% inert ingredients. Following the 1-h acclimatization period, treatments were applied to each compartment by pouring the contents of the respective aliquot into the water column. Treatments were administered each day at ~0830 and ~1530 until embryos developed eyes. Eggs were exposed to an untreated control and 2.5, 5, 10, 15 and 20 mg L⁻¹ PAA in each trough (*N* = 4); PAA treatments were applied nominally from the stock solution, which was confirmed to be 35 g L⁻¹ PAA using a Peracetic Acid Vacu-vials Kit (CHEMetrics). Individual treatment concentrations were verified as above 45 s (chosen in preliminary assays) after treatments were applied.

Saprolegniasis occurred naturally, and a daily log was maintained to record fungal growth in each compartment. A fish pathologist verified the

saprolegniasis via microscopic examination based on the typical hyphal elements and mycelial development. Samples from the control treatments of each spawn were cultured and were identified by PCR amplification and sequencing of the rRNA gene internal transcribed spacer (ITS) regions (White *et al.* 1990; Straus *et al.* 2009b).

Healthy fry congregated in the corners of the compartment after hatching. Prematurely hatched fry (PMH) appeared deformed, showed a lack of normal mobility, were a light yellow colour instead of the golden brown associated with healthy fry and did not congregate in the corners. Healthy fry were siphoned from each compartment and preserved in 70% ethanol to be counted. Live PMH fry were counted immediately. Success of the treatment was determined by the percent of living fry that remained in each compartment at the end of the study. Animal care and experimental protocols were approved by the SNARC Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

The study was conducted in a randomized block design with four troughs ($N = 4$); similar weight portions from an individual spawn were used in each trough. The fry survival rate was analysed by the Proc GLIMMIX procedure (version 9.2, SAS Institute) for differences between control and treatments. The GLIMMIX procedure fits statistical models to data with correlations or non-constant variability and when the response is not necessarily normally distributed; these models are known as generalized linear mixed models (GLMM). A significant difference was indicated at $P < 0.05$. Percent PMH, fungal growth and pH change were analysed by ANOVA.

A separate study determined the pH recovery after addition of specific doses of PAA. Each

compartment contained similar weight egg portions as the study described above and was treated with an aliquot of PAA. An Orion Research 3-Star Plus Conductivity Portable Meter (Thermo Electron Corporation) was set to continuously record pH at 1-min intervals until pH had returned to near initial levels (≤ 1 h).

Results and discussion

Total alkalinity and total hardness (as CaCO_3) of the well water were 243 and 128 mg L^{-1} , respectively; pH was 7.7. Dissolved oxygen (DO) and water temperature were $59 \pm 3.4\%$ (mean \pm SD) saturation and 23.6 ± 0.1 °C, respectively. Peracetic acid concentrations after treatments were 98%, 91%, 87%, 83% and 80% of the expected dose at 2.5, 5, 10, 15 and 20 mg L^{-1} PAA, respectively, and this can be attributed to rapid dissociations of higher concentrations. Eye pigment developed in the embryos by the afternoon on day 5, and all treatments were stopped; hatching was complete by day 9.

Table 1 displays results from each treatment of the present study. The hatch rates of each replicate (indicated by SD) clearly show the variability within individual spawns; such variability is common in commercial hatcheries. Fungal growth was severe in the untreated controls (Fig. 1a); all treatments were significantly different from the controls ($P < 0.05$). The highest percent survival of hatched fry was with 5 mg L^{-1} PAA (63%) administered twice daily. The 2.5 mg L^{-1} PAA treatment had slightly less survival, but provides a higher margin of safety to the eggs, to hatchery personnel and is more economical; therefore, this was determined to be the suggested therapeutic treatment. The 10 mg L^{-1} PAA treatment resulted

Table 1 Treatment concentration, fry survival rate (mean \pm SD), statistical P value resulting from Proc GLIMMIX comparing each treatment with the control, percent of survival that was considered prematurely hatched (PMH) fry and mean fungal surface area (cm^2) in the study on the effectiveness of peracetic acid (PAA) in controlling fungus on catfish eggs ($N = 4$)

PAA (mg L^{-1})	% Survival		% PMH	Fungal area	pH change $N = 3$
	$N = 4$	P value ^a			
0	11.1 \pm 7.3		2.8 \pm 1.9	3.6 \pm 3.8	
2.5	60.0 \pm 21.5	0.0006	2.1 \pm 2.9	0.1 \pm 0.4	0.24 \pm 0.07
5	63.3 \pm 22.9	0.0003	4.8 \pm 5.2	0.2 \pm 0.6	0.40 \pm 0.03
10	61.8 \pm 9.9	0.0004	2.0 \pm 0.9	0.1 \pm 0.5	0.49 \pm 0.06
15	32.1 \pm 23.0	0.0660	5.5 \pm 3.4	0.4 \pm 0.9	0.69 \pm 0.06
20	0.0 \pm 0.0	0.3213	0.0 \pm 0.0	0.3 \pm 0.6	0.80 \pm 0.06

^aA significant difference was indicated at $P < 0.05$.

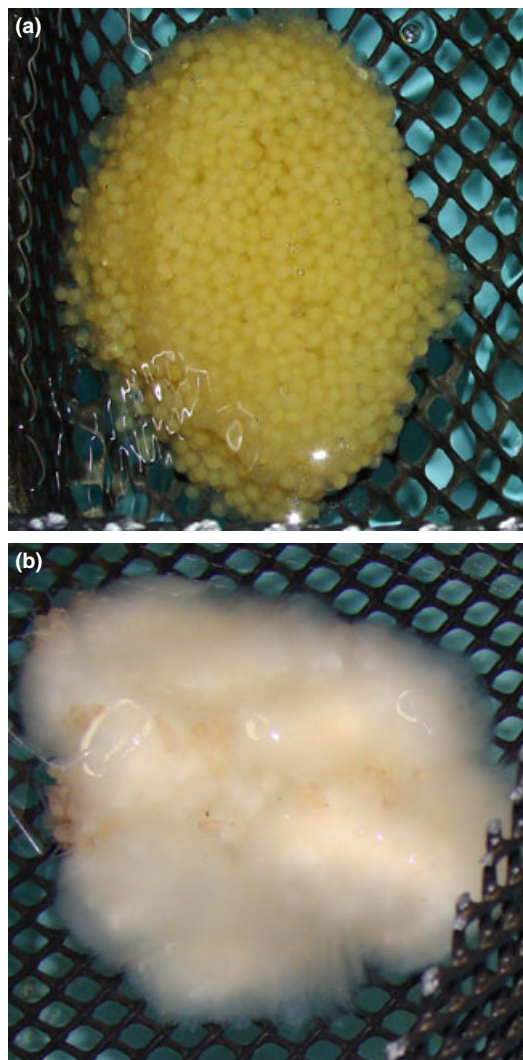


Figure 1 Channel catfish eggs: (a) eggs treated with peracetic acid and (b) untreated control eggs.

in comparable survival, but the margin of safety would be smaller, and it is not recommended. Recent research at SNARC has demonstrated that yolk sac fry are at least 1.6 times more tolerant of PAA than swim-up fry; therefore, continued treatments should be safe to hatching sac fry.

Very little fungus was present in treatments receiving 2.5 mg L⁻¹ PAA or higher (Fig. 1b), and concentrations of 15 and 20 mg L⁻¹ PAA were toxic to the eggs. The adhesive matrix in the 15 mg L⁻¹ PAA-treated compartments was beginning to dissolve by day 2, resulting in egg masses that were not tightly connected as normal; the outer eggs appeared swollen. The 20 mg L⁻¹ PAA-treated compartments exhibited a similar effect, but to a higher

degree, and resulted in complete mortality by day 4. Fungus samples were identified as *Saprolegnia* spp. through PCR and ITS sequence characterization. Two sequences were identified from the cultures; these sequences were 100% and 97% homologous with EU551153.1 and UNCW315, respectively, referenced in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). There was no apparent correlation of PMH fry to the untreated control or PAA treatment, except for the 20 mg L⁻¹ PAA treatment, and this can be attributed to toxicity. Preliminary observations at SNARC suggest that a portion of PMH fry may recover and appear normal. Future studies should investigate the survival of PMH fry for longer durations.

Mean survival of the 2.5, 5 and 10 mg L⁻¹ PAA treatments was 62% (Table 1). This is similar to what has been found in the commercial sector. The mean egg survival in the catfish industry is estimated to be ~60% (Wolters 2001) using formalin and iodine as needed, along with other optimal practices. However, in contrast to these therapeutants, PAA will degrade to harmless residues quickly.

As PAA alters pH levels, a profile of the rate of recovery was necessary. This pH profile indicates that treatments of 2.5, 5 and 10 mg L⁻¹ PAA returned to predosing levels by 40 min (Fig. 2); this would be equivalent to the time the eggs were exposed to PAA. The suggested therapeutic treatment of 2.5 mg L⁻¹ PAA caused pH to decrease by approximately 0.2 units, and this would not be detrimental to hatching (Table 1). Catfish are generally hatched in water that has sufficient buffering capacity similar to that used in the present study. Water with lower buffering capacity could cause pH levels to take longer to recover, so sample trials should be conducted before using PAA. Water chemistry should always be taken into consideration before treating to avoid losses. The 20 mg L⁻¹ PAA treatment caused the pH to decrease 0.8 units very rapidly and took longer to recover; this concentration was apparently enough active PAA to cause toxicity to the eggs and dissolve the adhesive matrix holding the eggs together. Also, the treatment with the highest percent survival of hatched fry (5 mg L⁻¹ PAA) had a significantly higher pH decrease than the 2.5 mg L⁻¹ PAA treatment; this is another reason to choose the lower concentration.

Marking *et al.* (1994) evaluated antifungal agents for fish culture and found PAA ineffective for control

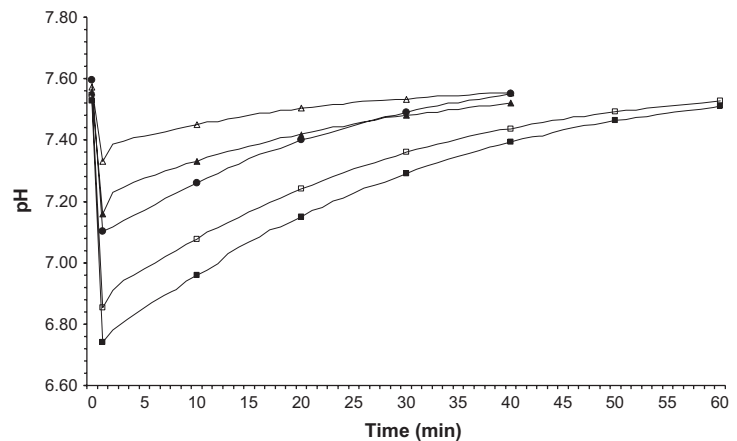


Figure 2 A profile of the rate of pH recovery after addition of an aliquot of peracetic acid (PAA) in flow-through water ($N = 3$). Final concentrations were 2.5 (Δ), 5 (\blacktriangle), 10 (\bullet), 15 (\square) and 20 (\blacksquare) mg L^{-1} PAA.

of fungus on rainbow trout, *Oncorhynchus mykiss* (Walbaum), eggs at 5 mg L^{-1} PAA and toxic at fungicidal concentrations. Meinelt *et al.* (2005) evaluated two PAA products for their ability to reduce fungus on rainbow trout eggs and determined that 3 mg L^{-1} PAA administered twice daily over 2-h periods worked best and is apparently well tolerated by the embryos. An *in vitro* study by Marchand *et al.* (in press) investigated the reduction in growth of *S. parasitica* when exposed to several commercial PAA products and found minimum inhibitory concentrations ranged from 4 to 10 mg L^{-1} PAA; no visible growth was observed in the Petri dishes when treated with the above concentrations, and growth was decreased in comparison with the control below these concentrations.

There are many commercial PAA products with varying ratios of acetic acid and hydrogen peroxide. The *in vitro* effectiveness of these products has been shown on several pathogens (Marchand *et al.* in press). The present study confirms that PAA demonstrates *in vivo* effectiveness; future studies should investigate the safety and define the toxicity of PAA to catfish fry. Studies should also investigate its effectiveness on other pathogens and in various water chemistries. The disinfectant properties of PAA should be useful to prevent bacterial growth on eggs, and this warrants further research. Alternate treatment regimens (i.e. additional treatments, static treatments, RAS) could be investigated to improve effectiveness of PAA.

These studies show that the use of PAA can reduce the growth of fungus on eggs of different fish species. However, different hatching methods and different treatment conditions require strategies to address the specific conditions and fish

species. For catfish in a flow-through system, the present research determined that using PAA gave 60% survival, which is comparable to hatching rates of 57% for formalin and 55% for copper sulphate (Mitchell *et al.* 2010). One advantage in using PAA is that it does not produce any residues that would harm fry or the environment. In aquaculture, several previously used chemicals have been banned because of harmful side effects (e.g. malachite green; Rintamäki-Kinnunen *et al.* 2005). The application of environmentally benign PAA treatment might be attractive to U.S. aquaculture.

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